Supporting Information

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SI Experimental Procedures

Animal Experiments. Mice were bred and housed in a pathogen-free facility following the protocol approved by the Animal Care Research Committee at Baylor College of Medicine. Chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) *COUP-TFII* lacZ knock-in mice, *COUP-TFII*$^{flox/flox}$ mice, and *COUP-TFII* heterozygous mice were generated and described previously (1). *ROSA26*$^{CRE\text{-}ERT2/+}$ mice harboring a tamoxifen-inducible CRE-ERT2 fusion under the control of ROSA26 promoter were crossed with *COUP-TFII*$^{flox/flox}$ mice to generate *ROSA26*$^{CRE\text{-}ERT2/+}$; *COUP-TFII*$^{flox/flox}$ mice. Tamoxifen (Sigma) was dissolved in corn oil and injected intraperitoneally into pregnant females (E11.5) at 2 mg per animal.

Cell Culture, Transfection, Differentiation, and Staining of Cultured Cells. C3H10T1/2 cells, obtained from the American Type Culture Collection, were cultured and induced to differentiate into adipocytes, osteoblasts, chondrocytes, and myoblasts as reported (2–4). Six- to eight-week-old *COUP-TFII*$^{flox/flox}$ mice and inducible *ROSA26*$^{Cre\text{-}ERT2/+}$; *COUP-TFII*$^{flox/flox}$ mice were injected with tamoxifen at 1 mg per 50 g of body weight for 3 consecutive days to ablate the *COUP-TFII* gene, and then primary bone marrow stromal cells (BMSCs) were isolated and amplified from wild-type and COUP-TFII knockout mice as described (5). Briefly, mice were killed, tibiae and femurs were removed, and excess soft tissue was eliminated. Marrow stromal cells were flushed from femurs and tibiae and passed through a 70-μm cell strainer to remove marrow chunk. The slurry was spun and cells were resuspended and plated in dishes containing α minimum essential medium (α-MEM) supplemented with 20% FBS. After 2 wk of culture and expansion, cells were harvested and plated for differentiation assay. Differentiation of BMSCs into adipocytes, osteoblasts, chondrocytes, and myocytes was performed following protocols reported previously (5, 6). Human adipose-derived mesenchymal stem cells (hAMSCs) were purchased from Invitrogen. Adipogenic and osteogenic differentiation of hAMSCs was conducted using the manufacturer's differentiation kit (Invitrogen).

COUP-TFII–specific siRNA and shRNA retroviruses were generated and transfected as reported (7). Briefly, C3H10T1/2 and human adipose-derived mesenchymal stem cells were transduced with retrovirus and cultured with puromycin at 1 mg/mL. Then, the stable pools of cells were transferred to media containing the appropriate stimuli to induce lineage-specific differentiation. Plates were stained with oil red O to to visualize lipid accumulation, alizarin red and von Kossa for intracellular calcium deposition, and alcian blue for chondrocyte formation, respectively.

Real-Time PCR, Western Blot, Immunoprecipitation, Reporter Analysis, and Chromatin Immunoprecipitation Assay. Total RNA was extracted, and DNase was treated and reverse-transcribed with random primers using SuperScript II reverse transcriptase (Invitrogen). Real-time PCR for genes of interest was performed with either inventoried TaqMan primer/probe mix (Applied Biosystem) or SYBR green-based RT-PCR. Levels of gene expression were normalized to the level of 18S rRNA or cyclophilin A. Primer information is available on request.

Total proteins were extracted from cells following standard protocols. Nuclear proteins were extracted using an NE-PER Kit (Pierce). The primary antibodies used in this study were as follows: COUP-TFII (R&D Systems), β-actin (Sigma), PPARγ (Chemicon), aP2 (R&D Systems), adiponectin (Affinity BioReagents), β-galactosidase (Biogenesis), myogenin (Abcam), myosin heavy chain (Sigma), Alpl (R&D Systems), Sox9 (Abcam), Runx2 (MBL), PARP (Millipore), cyclophilin A (Millipore), β-catenin (Santa Cruz Biotechnology), adenomatous polyposis coli (APC) (Santa Cruz Biotechnology), cyclin D1 (Sigma), and GSK-3β and p-GSK-3β (Cell Signaling). HRP-conjugated secondary antibodies were purchased from DAKO. Signals were visualized with a SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce). For immunoprecipitation, nuclear proteins were extracted from C3H10T1/2 cells and precipitated with Runx2 antibody. Dual luciferase assays were conducted as described by the manufacturer (Promega).

Chromatin immunoprecipitation assays were performed in C3H10T1/2 cells following the protocol provided by Millipore. Eluted DNA fragments were analyzed by PCR using the following primers: Wnt10b proximal COUP-TFII site, which was located 2 kb upstream of the transcription initiation site, forward (F) 5′-GTCAGGACCCAGGTCAGATC-3′ and reverse (R) 5′-TCCTGAATAGGTCTGTGGGG-3′; Wnt10b control primer, (F) 5′-GGAGGGGGGACGTGTAGCAAAA-3′ and (R) 5′-AGGCAAACATCAGGGGAGGG-3′; osteocalcin Runx2 site, (F) 5′-GAGAGCACACAGTAGGAGTGGTGGAG-3′ and (R) 5′-TCCAGCATCCAGTAGCATTT ATATCG-3′; Sox9 Sp1 site located 1.5 kb downstream relative to the transcription initiation site of annotated Sox9 gene, (F) 5′-CAGCAAGACTCTGGGCAAG-3′ and (R) 5′-CCAAGAGCAATCCCAAGAAC-3′.

Histology, in Situ Hybridization, and Immunohistochemistry. For immunohistochemistry, tissues or embryos were dissected, fixed, and processed as described (1, 7, 8). Immunostaining was carried out with a TSA Kit (Molecular Probes) following the manufacturer's protocol. X-gal staining of the cryostat sections was performed as previously reported (7, 8). In situ hybridization was performed with cryostat sections as described (9). Hematoxylin/eosin, oil red O, von Kossa, and alcian blue staining were performed using standard procedures.

Bone Histomorphometry and Microcomputerized Tomography Analysis. Femur and vertebrae were dissected and fixed in 4% paraformaldehyde, dehydrated in ethanol, and embedded in methyl methacrylate resin. von Kossa/van Gieson staining was performed for bone volume/tissue volume measurement. For microcomputerized tomography (μCT) analysis, femur bone specimens were dissected and soft tissues were carefully removed, and the samples were then fixed with 70% ethanol and scanned with a μCT scanner. μCT results were analyzed using the Stereology function of MicroView software (GE Healthcare).

Statistical Analysis. All data are expressed as mean ± SEM of representative experiments. Statistical calculations were performed by Student's t test, and $P < 0.05$ was considered statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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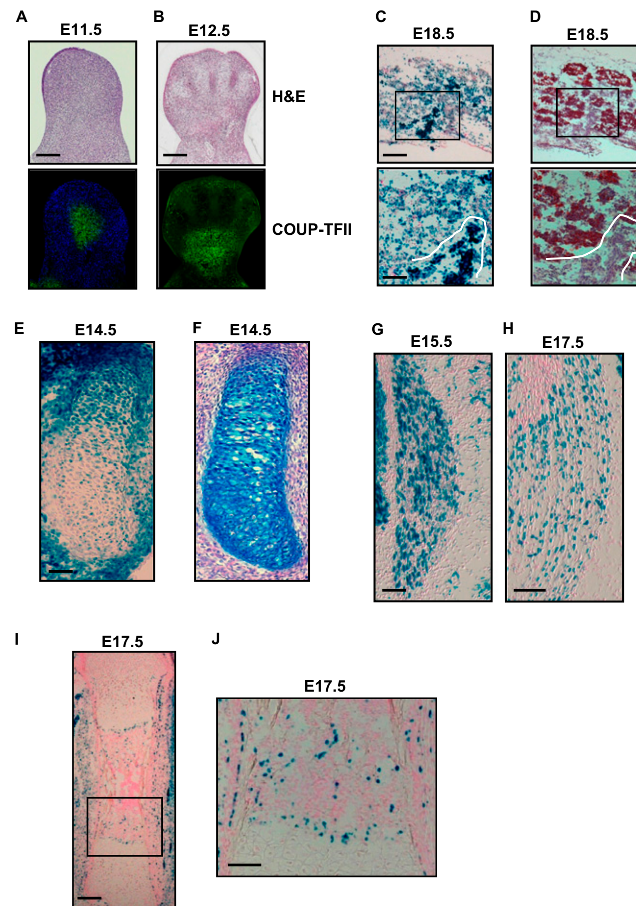

Fig. S1. Expression profiling of COUP-TFII gene indicates a role in mesoderm-derived tissue development. (*A* and *B*) H&E staining visualized the morphology of wild-type hindlimb at embryonic day (E)11.5 (*A*) and E12.5 (*B*). Immunohistochemical staining shows that COUP-TFII is expressed in the central core of the limb bud (*Lower*). [Scale bars, 100 μm (*A*) and 200 μm (*B*).] (*C* and *D*) Primitive white adipose tissues from E18.5 embryos were stained with oil red O (*D*), and COUP-TFII protein was detected in the fat tissue (*C*). [Scale bars, 100 μm (*Upper*) and 50 μm (*Lower*).] The boxed regions indicate the magnified areas (*Lower*). Solid lines indicate the strong X-gal-staining area. (*E* and *F*) COUP-TFII protein was detected in the chondrocytes as demonstrated by β-gal staining (*E*) and alcian blue staining (*F*) of E14.5 pelvis. (Scale bar, 50 μm.) (*G* and *H*) COUP-TFII protein was expressed in the muscle at different stages: E15.5 (*G*) and E17.5 (*H*). [Scale bars, 50 μm (*G*) and 100 μm (*H*).] (*I* and *J*) X-gal staining of E17.5 femora taken from *COUP-TFII* lacZ knock-in mice (*I*), and a higher magnification of the boxed regions is shown in *J*. [Scale bars, 200 μm (*I*) and 100 μm (*J*).]

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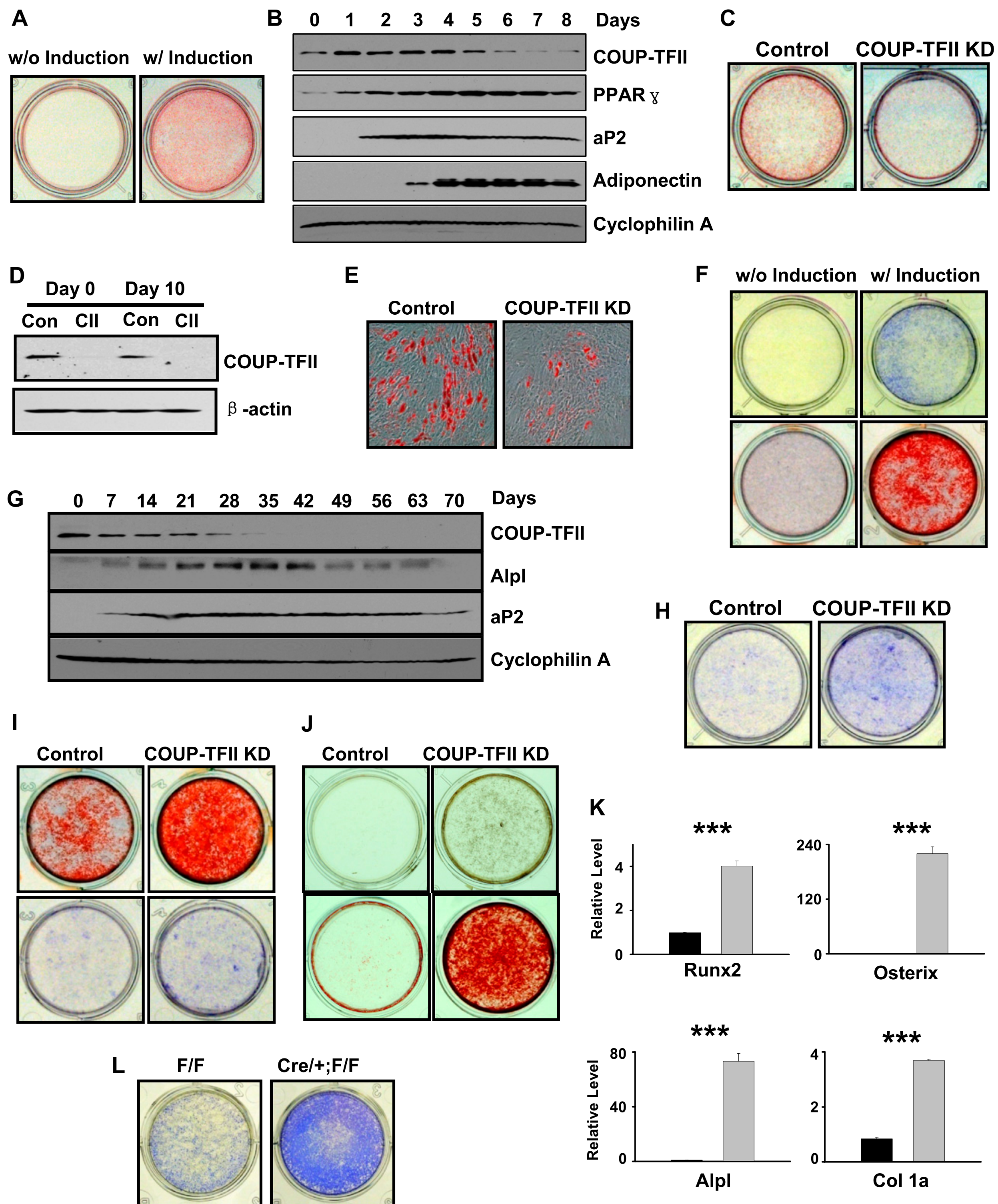

Fig. S2. COUP-TFII controls adipocyte and osteoblast differentiation. (*A* and *B*) C3H10T1/2 cells were incubated with (*Right*) or without (*Left*) adipogenic stimuli for 8 d and stained with oil red O (*A*), and expression of COUP-TFII, PPARγ, aP2, adiponectin, and cyclophilin A was examined by immunoblots (*B*). (*C*) Control or COUP-TFII shRNA-treated C3H10T1/2 cells were transferred to adipogenic medium and stained with oil red O on day 6. (*D* and *E*) Inhibition of COUP-TFII protein in hAMSCs treated with COUP-TFII retrovirus (*D*) and reduction of COUP-TFII protein led to defects in lipid accumulation (*E*). (*F* and *G*) C3H10T1/2 cells were incubated in the presence (*Right*) or absence (*Left*) of osteogenic stimuli for 10 d and stained with alkaline phosphatase (ALP) (*F*, *Upper*) or for 60 d and stained with alizarin red (*F*, *Lower*). Expression profile of COUP-TFII, Alpl, and aP2 during osteoblastogenesis (*G*). (*H*) COUP-TFII–depleted C3H10T1/2 cells and control cells were stained with alkaline phosphatase for osteoblast activity on day 60 after culturing in osteogenic medium. (*I*) Alizarin red staining (*Upper*) and ALP staining (*Lower*) of C3H10T1/2 cells transfected with control siRNA (*Left*) or COUP-TFII–specific siRNA (*Right*) and analysis for osteoblast differentiation on day 60. (*J*) hAMSCs stably transduced with COUP-TFII retrovirus and control cells were subjected to von Kossa (*Upper*) or alizarin red (*Lower*) staining on day 42. (*K* and *L*) Gene expression analysis (*K*) and ALP staining (*L*) of bone marrow stromal cells undergoing adipogenesis for 8 d (*K*) and 12 d (*L*). Gray bars represent COUP-TFII KO BMSCs. ***$P < 0.001$.

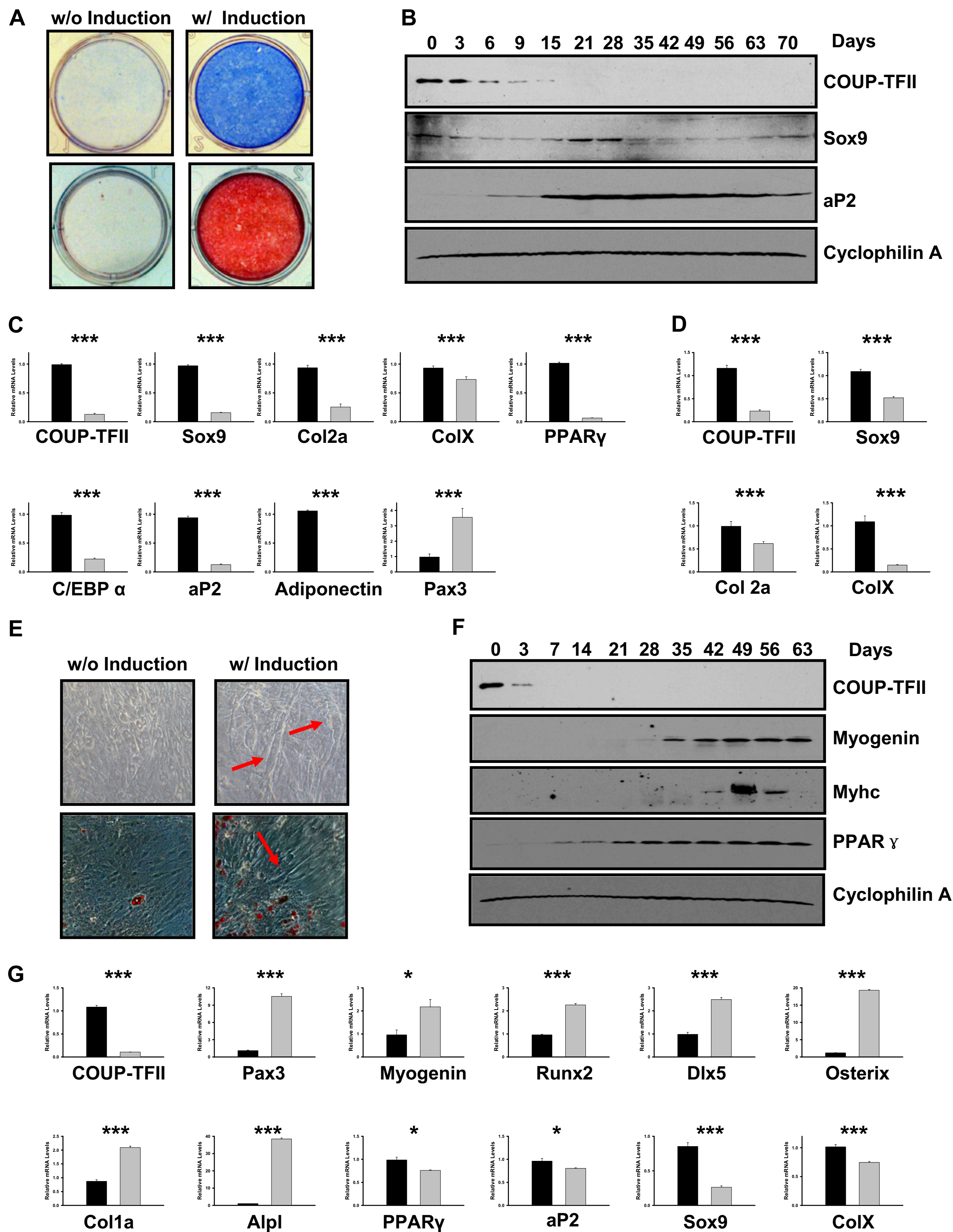

Fig. S3. COUP-TFII regulates chondrocyte and myocyte formation. (*A* and *B*) C3H10T1/2 cells were cultured in the presence (*Right*) or absence (*Left*) of BMP-2 for 35 d. Culture dishes were stained with alcian blue (*Upper*) or oil red O (*Lower*). Cells were collected to monitor COUP-TFII, Sox9, and aP2 protein during this process (*B*). (*C*) BMSCs obtained from wild-type and COUP-TFII mutant mice were subjected to chondrogenic stimuli. Gene expression analyses were performed at day 10. (*D*) Micromass culture of BMSCs obtained from COUP-TFII KO mice and wild-type mice and chondrogenic markers were examined at day 14. (*E* and *F*) C3H10T1/2 cells convert into myoblasts (*Upper*) or adipocytes (*Lower*) in medium containing 5-azacytidine (*E*) and the expression of COUP-TFII, myogenin, Myhc, and PPARγ was analyzed by Western blot (*F*). Arrows indicate multinucleated myotubes. (*G*) Gene expression profile of lineage-specific markers of BMSCs cultured with 5-aza-cytidine for 18 d. Gray bars represent COUP-TFII–deficient cells (*C*, *D*, and *G*). Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$.

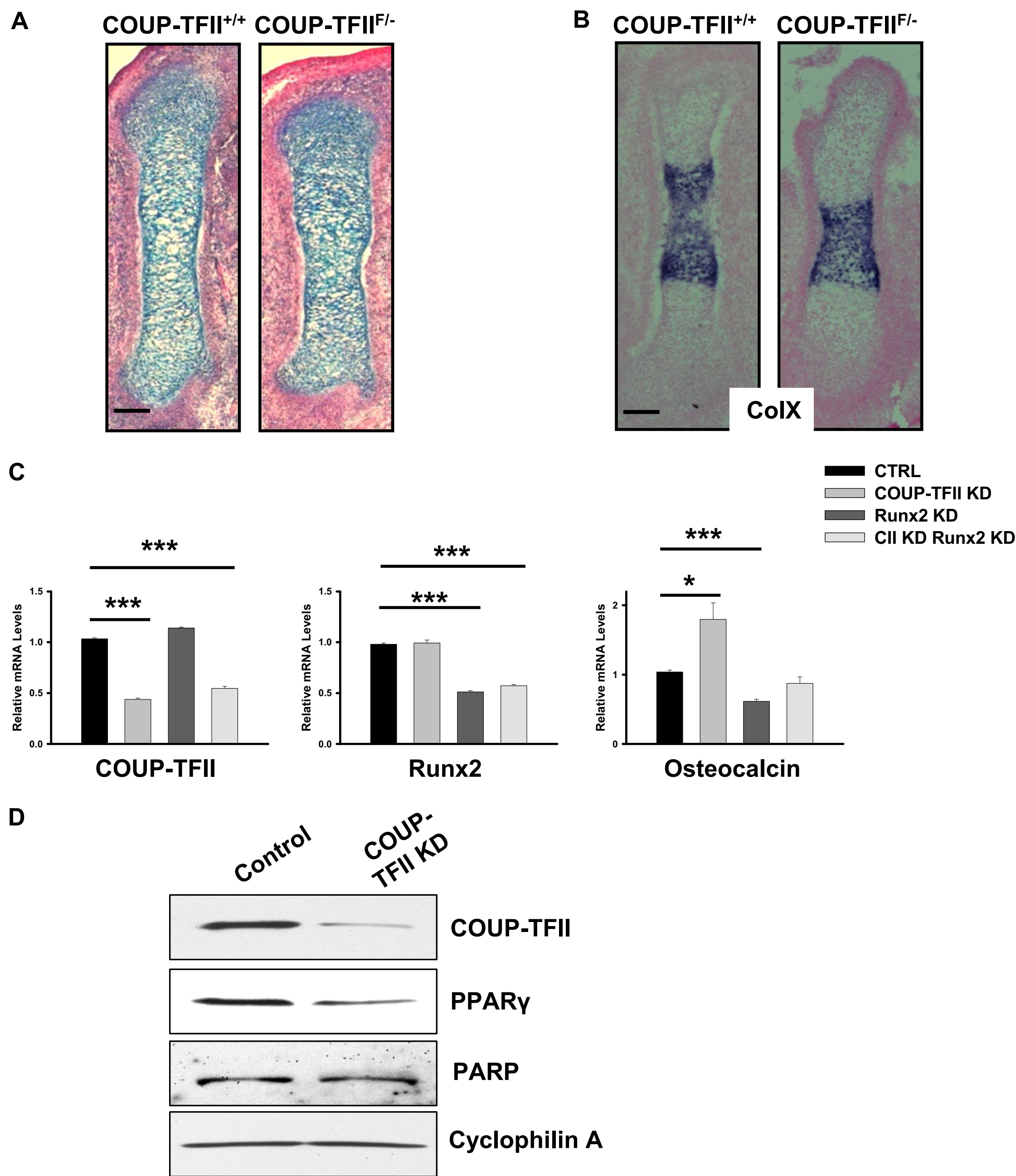

Fig. S4. COUP-TFII–deficient mice display cartilage development defects, and COUP-TFII modulates Runx2 activity and PPARγ expression. (*A* and *B*) E14.5 femurs of wild-type and COUP-TFII hypomorphic mutant mice were stained with alcian blue (*A*) and in situ hybridization (ISH) with ColX probe (*B*). (Scale bars, 100 μm.) (*C*) C3H10T1/2 cells were treated with siRNA against COUP-TFII, Runx2, or both. Cells were then cultured under osteogenic conditions for 4 d and RNA was extracted for quantitative PCR analysis. (*D*) Depletion of COUP-TFII inhibits PPARγ expression, as shown by Western blot of nuclear extract from C3H10T1/2 cells. $^{*}P < 0.05$, $^{***}P < 0.001$.